Microbial Ecology

Analysis of Soil Whole- and Inner-Microaggregate Bacterial Communities

D.L. Mummey and P.D. Stahl

Department of Renewable Resources, University of Wyoming, P.O. 3354, Laramie, Wyoming 82070, USA

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Abstract

Although soil structure largely determines energy flows and the distribution and composition of soil microhabitats, little is known about how microbial community composition is influenced by soil structural characteristics and organic matter compartmentalization dynamics. A UV irradiation-based procedure was developed to specifically isolate inner-microaggregate microbial communities, thus providing the means to analyze these communities in relation to their environment. Whole- and inner-microaggregate fractions of undisturbed soil and soils reclaimed after disturbance by surface coal mining were analyzed using 16S rDNA terminal restriction fragment polymorphism (T-RFLP) and sequence analyses to determine salient bacterial community structural characteristics. We hypothesized that inner-microaggregate environments select for definable microbial communities and that, due to their sequestered environment, inner-microaggregate communities would not be significantly impacted by disturbance. However, T-RFLP analysis indicated distinct differences between bacterial populations of inner-microaggregates of undisturbed and reclaimed soils. While both undisturbed and reclaimed inner-microaggregate bacterial communities were found dominated by *Actinobacteria*, undisturbed soils contained only *Actinobacteridae*, while in inner-microaggregates of reclaimed soils *Rubrobacteridae* predominate. Spatial stratification of division-level lineages within microaggregates was also evidenced, with *Proteobacteria* clones being prevalent in libraries derived from whole microaggregates. The fractionation methods employed in this study therefore represent a valuable tool for defining relationships between biodiversity and soil structure.

Correspondence to: D.L. Mummey; E-mail: dan.mummey@mso.umt.edu

Introduction

Soil *Actinobacteria* aggregates are heterogeneous assemblages of organic and mineral particles operationally distinguished by size as macro- (>250 μm) and microaggregates (<250 μm) [28]. Macroaggregates are formed by temporary associations of microaggregates, minerals, and particulate organic matter, predominantly through enmeshment by fungal hyphae and plant roots [19]. Water-stable microaggregates, on the other hand, typically form by microbially mediated processes within macroaggregates and are largely dependent upon persistent organic binding agents for structural stability [28]. Soil physical disturbance generally results in decreased macroaggregate stability and the release of relatively stable microaggregates, which may then become building blocks for the next cycle of macroaggregate formation [19, 23–25].

Microaggregate interior regions may constitute a relatively definable habitat for microorganisms characterized by low predation, relatively stable moisture availability, and steep diffusional gradients resulting in low nutrient and oxygen availability [1, 7, 8, 21]. When compared to microaggregate outer surfaces or macroaggregates as a whole, microaggregate interiors likely represent a relatively stable and secluded habitat for microorganisms, especially after physical disruption of macroaggregate structure. It is also likely that microorganisms inhabiting the interior of microaggregates contribute relatively little to overall measurable microbial activity [4, 6], yet they may play key roles in ecosystem functioning based on metabolic strategies possible in anoxic environments and by aiding in aggregate formation and stabilization. It also seems likely that, because of their isolation, organisms occupying these microhabitats are much less amenable to manipulation than microorganisms on aggregate surfaces [8]. Defining populations associated with these niches is important to understanding how management practices influence soil mi-

crobial diversity, activity, and function, and to identify segments of the microbial population important to a given ecological process.

In this study we employ a soil fractionation procedure that utilizes high-energy UV irradiation to destroy outer-microaggregate microbial populations, thereby allowing for examination of physically protected inner-microaggregate bacterial populations. A primary objective of this study was to compare the bacterial diversity of whole- and physically protected inner-microaggregate communities. We hypothesized that, because of their sequestered environment, inner-aggregate bacterial communities are relatively stable and similar between undisturbed and reclaimed ecosystems.

Methods

Site and Soils. This study utilized soils collected at the Dave Johnson Coal Mine near Glenrock, Wyoming (43°N, 105°W). The landscape is a semiarid shrub-steppe with mean annual precipitation of 28 cm. The area has a rolling topography with a mean elevation of 1676 m. Undisturbed soils in the area are classified as Ustic Haplargids.

Soils were collected under western wheatgrass (*Agropyron smithii*) (5–10 cm depth) from two undisturbed sites (C98 and C95) and from sites reclaimed 11 and 17 years before analysis (R11 and R17, respectively). Treatment of soil on the reclaimed sites followed standard practice on surface mine sites; topsoil was removed before mining and stockpiled until mining operations were completed, on this site for over 10 years. Reclamation involved spreading stored soil onto overburden materials to a uniform depth (30 cm), tillage, and seeding with native plant species.

Soil Fractionation. Field-moist soils were immersed in deionized water on top of a 2000-, 250-, and 53-μm mesh screens and gently shaken. Microaggregates flushed through 2000- and 250-μm mesh screens are retained on a 53-μm sieve. Immediately following microaggregate isolation, subsamples for analysis of whole-microaggregate bacterial communities were placed in storage at −20°C. To isolate inner-microaggregate fractions, high-energy UV irradiation was employed to photo-oxidize organic matter [27], including nucleic acids of outer-microaggregate microbial communities. Our photo-oxidation system (Fig. 1) utilizes a horizontally aligned 450 W mercury vapor UV bulb encased in a cooling water-jacket as a radiation source. Quartz tubes (50 ml) containing aqueous microaggregate suspensions were fixed to a vertically aligned rotating wheel centered on the UV lamp. Tube rotation ensures that microaggregates remain suspended, without excessive abrasion, and are irradiated evenly from all angles. Because UV irradiation passing through mineral materials is negligible, only organic materials that aren't physically protected by mineral materials are photo-oxidized [27]. Subsets of all samples were exposed to UV irradiation for 24 hours. To determine the affect of different UV irradiation times, additional subsets of microaggregates from undisturbed soils C95 and C98 were treated for 12 hours.

Efficacy of the reactor was tested by adding *Bacillus subtilus* spores to quartz tubes containing microaggregates and 50 mL H_2O prior to irradiation. Irradiated and nonirradiated microaggregates were then analyzed for *Bacillus subtilus* 16S rDNA by terminal-restriction fragment length polymorphism (T-RFLP) analysis (see below). The ability of the system to destroy surface populations was also assessed by microscopically determining DAPI-stained surface cell numbers before and after irradiation.

DNA Extraction and PCR Amplification. DNA was extracted from approximately 0.4 g of each soil fraction using the UltraClean soil DNA kit (MoBio, Solano Beach, CA) according to the manufacturer's instructions. DNA concentrations in each sample were estimated using ethidium bromide–stained agarose gels with a pUC 18 molecular weight marker (Sigma, St. Louis, MO) as a calibration standard.

Amplification of template DNA was performed by using eubacterial 16S rDNA primers 46f (5′-GCYTAACACATGCAAGTCGA) and 536r (5′-GTATTACCGCGGCTGCTGG). Reaction mixtures contained 5 ng template DNA, 1× PCR buffer, 0.6 mM deoxynucleoside triphosphates, 3.5 mM $MgCl_2$, 0.2 μM each primer, and 1 U Taq DNA polymerase (Promega, Madison, WI). Amplification for T-RFLP analysis utilized 5′-FAM-labeled 46f (Applied Biosystems Inc, Fremont, CA). Amplifications were started with an initial denaturation step of 94°C for 3 min; 30 cycles of 94°C for 2 min, 48.5°C for 1 min, and 72°C for 1 min; cycling was completed with a final extension period of 72°C for 10 min. Thermocycling was conducted on an iCycler thermal cycler (Bio-Rad, Benecia, CA).

T-RFLP. Approximately 50 ng PCR product was digested in 20-μL reaction volumes with 6 U *Hha*I (New England Biolabs, Beverly, MA) for 2 h in the manufacturer's recommended reaction buffer. Digests were then purified by passage through gel filtration cartridges (Edge Biosystems, Gaithersburg, MD) and subsequently lyophilized. Three replicates from each sample were resuspended in 2 μL deionized formamide, 0.5 μL loading buffer, and 0.5 μL Genescan 500 ROX (Applied Biosystems Inc) size standards, denatured at 96°C for 2 min, then placed on ice until analysis. Terminal restriction fragments (T-RFs) within each sample replicate were separated by electrophoresis on denaturing 5% poly-

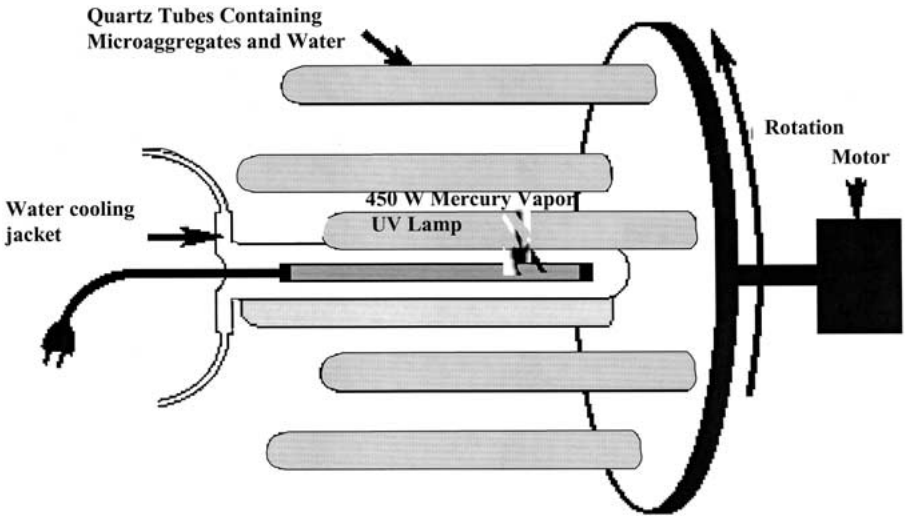

Figure 1. Schematic of photo-oxidation system. Microaggregates (0.2 g) are placed in horizontally oriented 50-mL DI-H_2O in quartz tubes. Rotation around a 450 W mercury vapor UV lamp gently suspends microaggregate particles while tubes are irradiated from all sides.

acrylamide gels (6 M urea) using an ABI 377 DNA sequencer. Sizes of between 50 and 500 bp were identified using Genescan analytical software (Applied Biosystems Inc). To quantify electropherogram output, a baseline threshold value of 50 fluorescence units, Local Southern size matching, and heavy smoothing were used.

Replicate profiles were aligned and standardized using methods similar to those of Dunbar et al. [2]. Briefly, relative fluorescence of replicate profiles was standardized to the smallest quantity by proportionally reducing each peak area in larger profiles. After proportional reduction of larger profiles, peaks having fluorescence values less than the threshold value were eliminated. Peaks not appearing in all replicate profiles were also eliminated.

Comparison of T-RFLP profiles from different samples requires standardization of relative fluorescence between samples [2]. Therefore, samples exhibiting greater total relative fluorescence were proportionally reduced as above and peaks having fluorescence values less than the threshold value were eliminated.

To determine relative similarities between T-RFLP profiles derived from each site and reaction, a similarity matrix was constructed based on Jaccard coefficients, enabling construction of a dendrogram using the UPGMA (unweighted pair group with mathematical averages) method. These analyses were carried out using SPSS v.10.1 software (SPSS Inc, IL).

Cloning and Sequencing. PCR products generated from whole-soil-community genomic DNA were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.). Randomly selected positive clones were screened for recombinant plasmids having inserts of expected size using agarose gel electrophoresis. Plasmids were purified from overnight cultures using Wizard Plus minipreps (Promega). Putative 16S rDNA inserts were sequenced using approximately 1 μg of purified plasmid DNA as the template in cycle sequencing reactions with fluorescent dye–labeled terminators (ABI PRISM dRhodamine Cycle Sequencing Ready Reaction Kit, Applied Biosystems Inc., Fremont, CA) according to the manufacturer's recommendations. Sequencing reactions were analyzed using a ABI 377 DNA sequencer (Applied Biosystems Inc). Sequences are deposited in GenBank under accession numbers AY186795 to AY186880.

Assignment of Cloned Sequences to Established Phylogenetic Divisions. In order to determine approximate phylogenetic affiliation, cloned 16S rDNA sequences were initially compared to reference sequences contained in the Genbank database using FASTA (San Diego Supercomputer Center). Sequences with <90% similarity to sequences of cultured organisms were screened for the presence of chimeric artifacts using the CHIMERA_CHECK program (version 2.7) [17]. Cloned 16S rDNA sequences were then aligned with 16S rDNA reference sequences using the ARB package (O Strunk, W Ludwig, Technical University of Munich, Germany) (http://www.mikro.biologie.tu-muenchen.de) and refined manually. Ambiguous positions were excluded from similarity calculations [14].

Evolutionary distance (neighbor joining with Kimura two-parameter correction with empirically determined base frequencies and empirically determined gamma distribution models of site-to-site rate variation) and maximum parsimony (default settings, heuristic search) methods were then employed to generate tree topologies using the software package PAUP* [D. L. SWOFFORD, Phylogenetic Analysis Using Parsimony (* and other methods). Version 4. Sinauer Associates, Sunderland, MA, 1998]. The robustness of tree topologies was determined by bootstrap resampling (1000 repetitions).

Results

Photoreaction. Although samples spiked with *B. subtilus* spores that were not exposed to UV radiation yielded high relative flourescence values for the anticipated *B. subtilus* T-RF size, photoreaction for 24 h reduced signals corresponding to *B. subtilus* to below detection limits. Although microaggregate-sized particles remained after photoreaction, no DAPI stainable cells were detected on aggregate surfaces, demonstrating that photo-oxidation of surface populations was complete.

Increasing the time microaggregates were exposed to UV radiation decreased the number of T-RFs detected (Fig. 2). Undisturbed whole microaggregates yielded 34 and 39 T-RFs, whereas 29 and 30 T-RFs were detected in samples exposed to UV radiation for 12 h, and 16 and 24 T-RFs were detected in samples treated for 24 h.

T-RFLP Analysis. A total of 56 T-RFs were found in whole microaggregates and microaggregates exposed to UV radiation for 24 h. Undisturbed whole-microaggregate samples yielded greater numbers of T-RFs (39 and 34 for C98 and C95, respectively) than did reclaimed whole-microaggregate samples (26 and 28 for R11 and R17, respectively). Fewer T-RFs were found for inner-microaggregate samples than for whole-microaggregate samples of all sites, with the lowest number in the most recently reclaimed soil (24, 16, 13, and 21 for C98, C95, R11, and R17, respectively).

Cluster analysis of T-RFLP profile similarity values of all samples revealed that bacterial community structures separate into two major groups, undisturbed and reclaimed samples (Fig. 3). Within undisturbed and reclaimed samples, bacterial community structures of whole and inner microaggregates clustered separately. Whole-microaggregate bacterial community structures exhibited relatively greater similarity than did inner microaggregates for both undisturbed and reclaimed soils.

Sequence Analysis. Partial sequences were obtained for 86 16S rDNA clones derived from whole and inner microaggregates of reclaimed and undisturbed soils. Based on RDP chimera check and other analyses, seven cloned sequences were found to be likely chimeric and discarded from further analysis. Nine division-level lineages were found, including representatives of candidate division TM7 [10] and the recently described *Gemmimonas* group (Sekiguchi et al., in review), formerly candidate division BD [10] (Fig. 4). *Chloroflexi* (two clones), *Verrucomicrobiales* (two clones), and *Gemmimonas* (one clone) were found only in inner microaggregates (Fig. 4). Candidate division TM7 (three clones), *Cyanobacteria* (four clones), and an unclassified group (two clones) were found only in whole-microaggregates of undisturbed soil, while *Acidobacterium* representatives (three clones) were found in whole-microaggregates of both reclaimed and undisturbed soils. A single *Nitrospira* representative was obtained from whole-microaggregates of reclaimed soil.

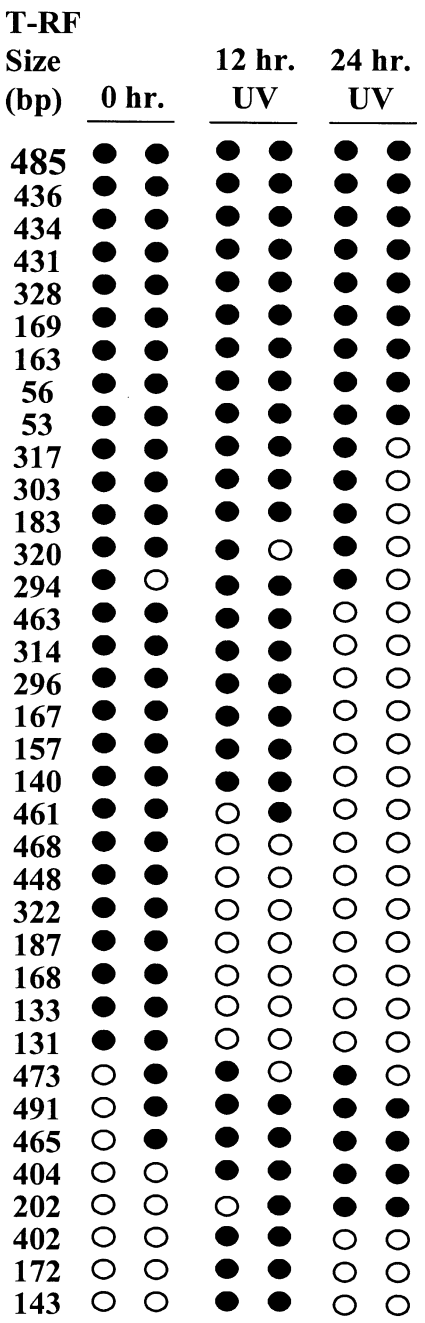

Figure 2. Terminal restriction fragment sizes found in undisturbed microaggregates exposed to UV radiation 0, 12, and 24 h. Filled circles indicate that a given T-RF size was detected and open circles indicate the absence of a given T-RF size. T-RFs found in only a single sample were removed for clarity.

Our analyses indicate that *Proteobacteria* (Fig. 5) and *Actinobacteria* (Figs. 6 and 7) are the predominant division-level bacterial lineages in the microaggregate environment of both undisturbed and reclaimed soils.

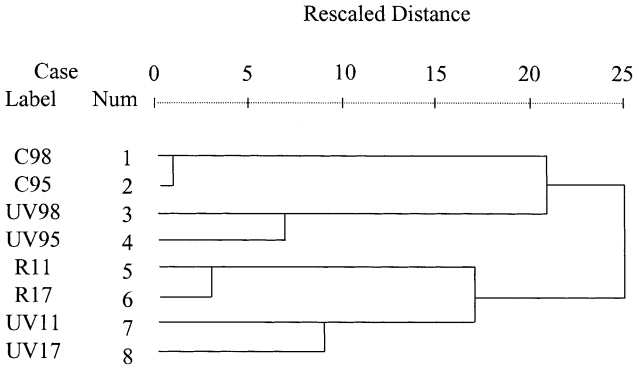

Figure 3. UPGMA dendrogram generated from T-RFLP profiles of undisturbed whole (C98 and C95) and inner (UV98 and UV95) microaggregates, and reclaimed whole (R11 and R17) and inner (UV11 and UV17) microaggregates.

However, our results strongly suggest that *Proteobacteria* predominate the outer-microaggregate environment, while *Actinobacteria* representation is greatest in microaggregate interiors. *Proteobacteria*, predominantly α-*Proteobacteria*, comprised 57% and 70% of whole microaggregate clones from undisturbed and reclaimed soil libraries, respectively, but accounted for only 30% of undisturbed inner-microaggregate clones and were absent from the reclaimed inner-microaggregate clone library (Fig. 8).

An inverse relationship was found for *Actinobacteria*. This bacterial division accounted for 6% and 50% of whole- and inner-microaggregate clones, respectively, from undisturbed soils (Fig. 8). Similarly, *Actinobacteria* represented 7% and 90% of whole- and inner-microaggregate clones, respectively, obtained from reclaimed soil.

Discussion

The location of bacteria within the soil matrix is a key factor affecting their survival, activity, and ecological function. Determining bacterial community structure in spatial relation to soil structure is therefore fundamental to elucidation of ecological function [21]. A number of studies have reported differences in bacterial populations associated with different soil particle size or aggregate fractions [1, 7, 8, 21, 22], including populations associated with subfractions of microaggregates [20]. However, the methods employed in these studies either are best suited to macroaggregates or disrupt microaggregate structure, making the characterization of inner-microaggregate bacterial community structure problematic.

A primary objective of this study was to determine whether inner-microaggregate bacterial communities represent definable and distinct populations within the soil matrix. To accomplish this, a photo-oxidation system was developed to selectively destroy microaggregate surface populations, thus allowing for isolation of inner-microaggregate bacterial communities. Our results clearly demonstrate spatial stratification of division-level lineages in relation to inner- and outer-microaggregate locations. *Actinobacteria* were highly abundant in inner microaggregates of the undisturbed soils (50% of sequenced clones) and are the dominant division-level lineage within microaggregates of the reclaimed soils (90% of sequenced clones) (Fig. 8). Although *Actinobacteria* appear to be a dominant inner-microaggregate lineage, the overall composition of undisturbed and reclaimed inner-microaggregate bacterial communities differed greatly. All undisturbed soil *Actinobacteria* representatives were associated with subdivision *Actinobacteridae* (Fig. 6), while *Rubrobacteridae* made up 53% of Actinobacterial clones sequenced from interiors of reclaimed soil microaggregates (Fig. 7). Very few *Rubrobacteridae* have been cultivated, and the majority (80%) of *Rubrobacteridae* found in this study are affiliated with phylogenetic group 3 [9], which has no cultivated representatives. Although little is known about this phylogenetic group, representatives have been shown to be relatively abundant in soils of a soybean field [29] and a grassland [18] and were recently found to be abundant in earthworm casts [3].

Reasons for the apparent shift from *Actinobacteridae* to *Rubrobacteridae* in reclaimed soil is unclear, although the shift could be the result of a number of factors that could potentially exert selective pressures on inner-microaggregate populations. The presumably stable inner-microaggregate environment may have very slow microbial turnover rates, and *Rubrobacteridae* populations could therefore be a carry over from soil storage. Both reclaimed soils analyzed in this study were stockpiled for a number of years prior to reclamation, and inner stockpiles are typically oligotrophic environments with very low plant-derived carbon inputs [5]. Additionally, gas diffusion into soil stockpiles is also likely low [5], potentially resulting in anoxic conditions, and consequent chemical changes would be expected to influence bacterial community structure. Mixing of surface and subsurface horizons that may have occurred during the topsoil salvage and reclamation process could also have contributed to observed differences in bacterial community structures. The degree to which inner-microaggregate bacterial community composition changes with depth is unknown, and populations associated with subsurface horizons may contribute significantly to population structure of reclaimed soil.

Verrucomicrobium are highly abundant in many soils, suggesting their ecological importance [15]. In this study, although only two *Verrucomicrobium* clones were obtained, both were from inner microaggregates. Although this bacterial division is represented by few cultured isolates, all soil isolates are ultramicrobacteria (0.1 μm^3 in volume) that preferentially use sugars as growth sub-

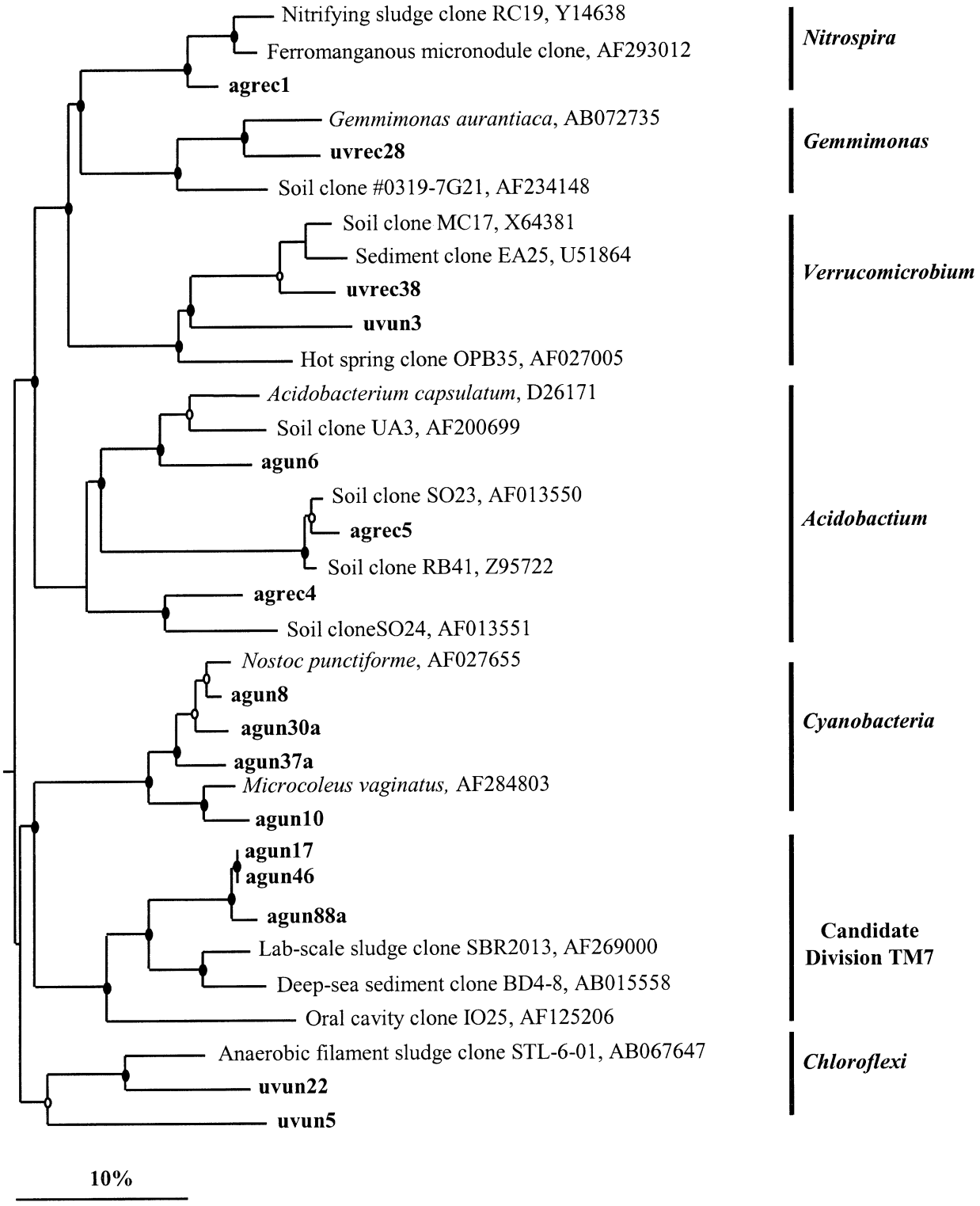

Figure 4. Evolutionary distance dendrogram of non-*Proteobacteria* or -*Actinobacteria* 16S rDNA clones obtained from inner and whole microaggregates of undisturbed and reclaimed soils. Branch points by bootstrap values >75% from both ED and MP methods are indicated by closed circles and open circles indicate bootstrap support of 50–74%. Branch points without circles were not resolved (bootstrap values: <50%). Clones sequenced in this study are in bold. Whole- and inner-microaggregate clones are represented by ag and uv, respectively, and undisturbed and reclaimed clones are represented by un and rec, respectively.

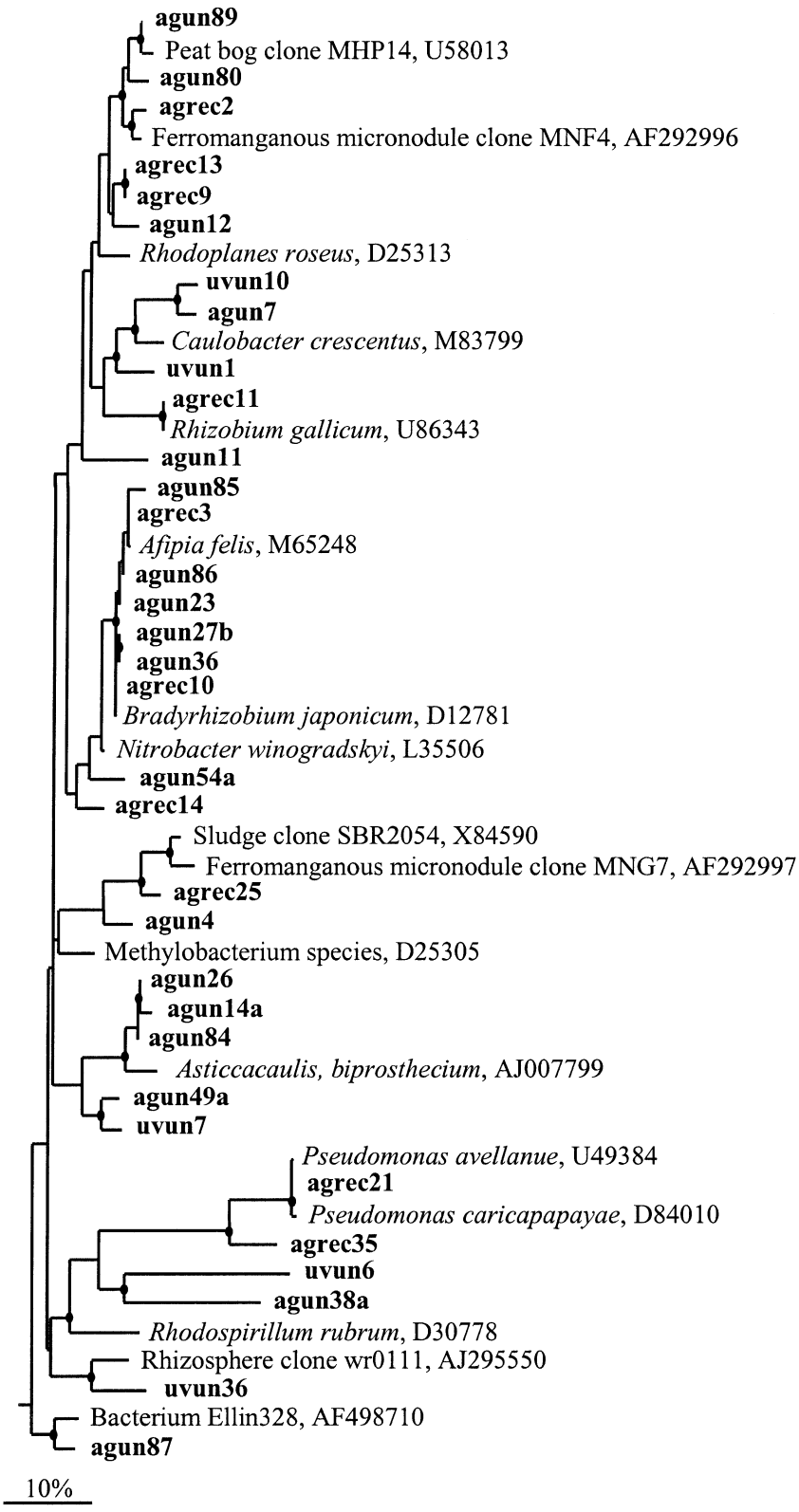

Figure 5. Evolutionary distance dendrogram of clones associated with the *Proteobacteria* phylum. Notation is as described for Fig. 3.

strates [11]. Small size and simple metabolism may be advantageous for life in the inner-microaggregate environment.

In contrast to the inner-microaggregate clone libraries, *Proteobacteria* were the dominant lineage among clones obtained from whole microaggregates, repre-

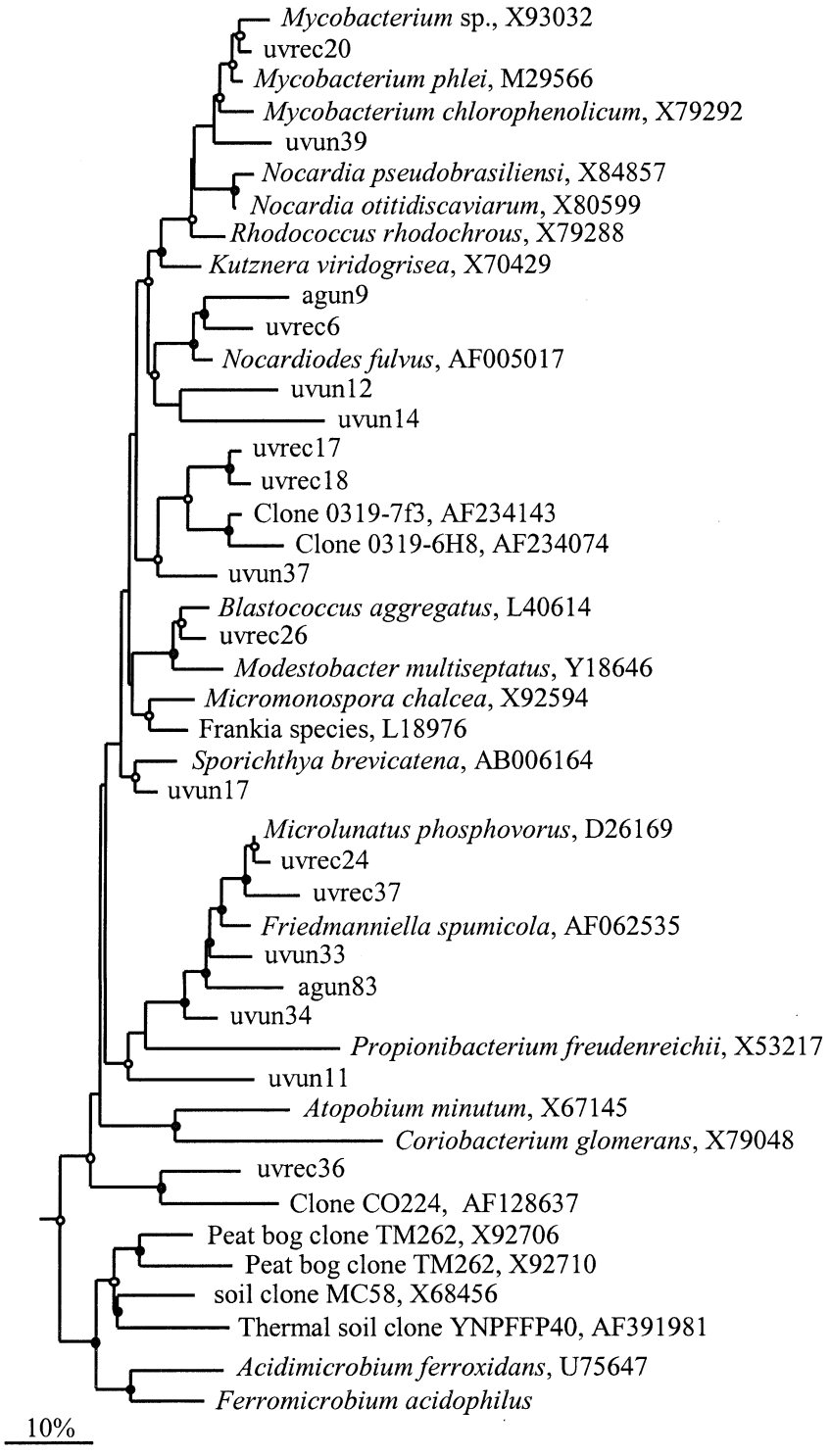

Figure 6. Evolutionary distance dendrogram of clones associated with the *Actinobacteria* phylum. Notation is as described for Fig. 3.

senting 56% and 71% of clones obtained from whole microaggregates of undisturbed and reclaimed soils, respectively. The disparity between *Proteobacteria* representation among inner- and whole-microaggregate clones, especially in reclaimed soils, suggests that these populations are predominantly located near or on microaggregate surfaces. A number of clones exhibiting high homology to common rhizosphere species, including potential plant symbiont species of the *Rhizobium* group, were obtained from whole microaggregates of both undisturbed and reclaimed sites, but were absent in inner-microaggregate clone libraries. Clones affiliated with *Cyanobacteria*, including chloroplasts, were also obtained exclusively from whole microaggregates,

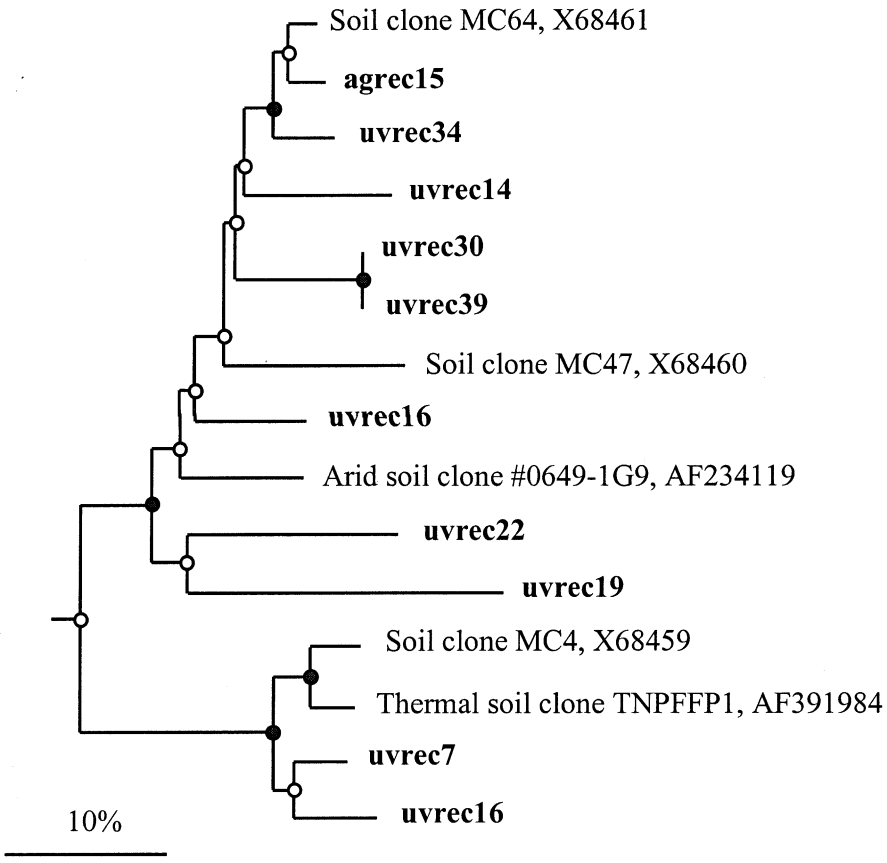

Figure 7. Evolutionary distance dendrogram of clones associated with *Actinobacteria* subdivision *Rubrobacteria*. Notation is as described for Fig. 3.

further suggesting that bacterial populations inhabiting microaggregate surfaces exhibit greater similarity to rhizosphere bacterial communities than populations of inner microaggregates. In addition, candidate division TM7 representatives were also obtained exclusively from whole microaggregates, suggesting an outer surface niche.

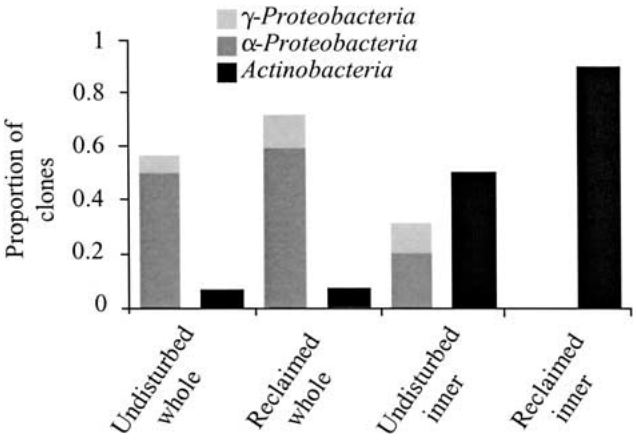

Figure 8. Relative abundance of *Proteobacteria* and *Actinobacteria* affiliates within sequenced clones of whole and inner microaggregates of undisturbed and reclaimed soils.

Conclusion

The results of this study show that fractionation of soil microaggregates by UV irradiation is a valuable tool for comparing bacterial community structures at an ecologically relevant microscale level. Our results clearly indicate relationships between bacterial community structure and soil microscale spatial organization. This microscale bacterial diversity raises questions about interactions between bacterial populations inhabiting the different environments and how these interactions influence observable ecological function.

Fractionation methods, such as employed in this study, should make it possible to locate sites where specific soil processes take place and, conversely, sites where such reactions are unlikely. At present, activities of inner-microaggregate bacterial populations are unknown. Rigorous characterization of the inner-microaggregate chemical and physical environment, in conjunction with bacterial community analyses, should provide insights into functional relationships. In addition to community structure, analysis of functional genes within these populations will greatly increase our comprehension of the role these populations play in soil function. The potential role of inner-microaggregate microbial communities in formation of soil structure, and decomposition and

compartmentalization dynamics of organic materials, warrants further study.

Many of the clones obtained from inner-microaggregate samples in this study are similar to organisms widely distributed in soils, but for which few or no phenotypic data are available. Knowledge of diversity patterns within the soil matrix will greatly aid in determining ecological function. This knowledge will be useful to interpretation of bulk soil analyses, potentially leading to diagnostic measures of soil health and function.

Acknowledgments

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